Spermatogenesis Protein

The present invention relates to a spermatogenesis protein, a DNA coding for such a protein and a method of producing such a protein. The invention also relates to antibodies directed against the protein and to the use of the DNA and the protein for studying or influencing spermatogenesis.

Spermatogenesis is referred to as the formation of sperms in mammals or humans. This formation takes place in the testicles. During spermatogenesis, in particular the pachytene stage of meiosis, the X and Y chromosomes accumulate and form what is called a sex body. The X and Y chromosomes are present inactively therein, i.e. they are not transcribed.

It has now turned out that some of the genes of the X and Y chromosomes have partner genes which are localized in autosomal manner. They are expressed *inter alia* in the testicles, so that their gene products have compensatory functions in spermatogensis as regards the corresponding inactivated genes of the X and Y chromosomes.

Influencing spermatogenesis is still a major problem. This is in particular due to the fact that spermatogenesis is not understood in detail.

It is thus the object of the present invention to provide a product by which spermatogenesis can be studied and possibilities can optionally be shown by means of which spermatogenesis can be influenced.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on applicant's findings that the gene XAP-5 localized in X-chromosomal manner has a partner gene which is localized in autosomal fashion and is expressed in many tissues. For example, the expression can be found in the testicles, it being here especially strong in spermatogenesis, in particular in the stages of primary and secondary spermatocytes as well as the round spermatocytes. The partner gene is referred to as X5L and is localized in the human genome on chromosome 6 in the region 6pter. Applicant isolated and characterized X5L on the PAC clone LLNLp 704K12294Q13. The DNA comprises a coding sequence and an intron and results in an about 1.6 kb long cDNA. It codes for an approximately 37 kD long spermatogenesis protein comprising 325 amino acids and referred to as X5L protein (cf. figures 1, 2 and 5, 6). Applicant also found out that mutations in the X5L protein may impair spermatogenesis.

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According to the invention applicant's findings are utilized to provide a spermatogenesis protein (X5L protein) comprising the amino acid sequence of figure 1 or an amino acid sequence differing therefrom by one or several amino acids, a homology of at least 70 % being present between the latter amino acid sequence and the amino acid sequence of figure 1.

The expression "an amino acid sequence differing by one or several amino acids comprises any amino acid sequence coding for an X5L protein and having a homology of at least 80 % with respect to that of figure 1. The amino acid sequence may differ from that of figure 1 by additions, deletions, substitutions and/or inversions of individual amino acids. In particular, the amino acid sequence may be that of figure 3.

Another subject matter of the present invention is a nucleic acid which codes for an X5L protein. The nucleic acid may be an RNA or a DNA, e.g. a cDNA. A DNA is preferred which comprises the following:

(a) The DNA of figure 1 or a DNA differing therefrom by one or more base pairs, the latter DNA hybridizing with the

DNA of figure 1 and coding for an X5L protein whose amino acid sequence has a homology of at least 80 % to that of figure 1, or

(b) a DNA related to the DNA of (a) via the degenerated genetic code.

The expression "a DNA differing by one or more base pairs" comprises any DNA sequence coding for an X5L protein, which hybridizes with the DNA of figure 1 and codes for an X5L protein whose amino acid sequence has a homology of at least 80 % to that of figure 1. The DNA sequence may differ from the DNA of figure 1 by additions, deletions, substitutions and/or inversions of individual base pairs. In particular, the DNA sequence may be that of figures 2 to 4. The expression "hybridization" refers to hybridization under common conditions, in particular at 20°C below the melting point of the DNA sequence.

The DNAs of figures 1 to 4 were deposited as h-X5L-c, h-X5L-g, m-X5L-c and m-X5L-g on November 26, 1998 with DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen* [Germany-type collection of microorganisms and cell cultures]) under DSM 12550, DSM 123553, DSM 12552 and DSM 12551, respectively.

A DNA according to the invention may be present as such or in combination with any other DNA. In particular, a DNA according to the invention which codes for an X5L protein may be present in an expression vector. A person skilled in the art is familiar with examples thereof. In the case of an expression vector for *E. coli* these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b, and pQE-8. For the expression in yeast e.g. pY100 and Ycpad1 have to be mentioned, while e.g. pKCR, pEFBOS, cDM8 and pCEV4 have to be indicated for the expression in animal cells. The bacculovirus expression vector pAcSGHisNT-A is particularly suitable for the expression in insect cells.

The person skilled in the art knows suitable cells to express the DNA according to the invention which is present in an expression vector. Examples of such cells comprise the *E. coli* strains HB101, DH1, x1776, JM101, JM 109, BL 21 and SG 13009, the yeast strain *Saccharomyces cerevisiae* and the animal cells L, NIH 3T3, FM3A, CHO, COS, Vero and HeLa as well as the insect cells sf9.

The person skilled in the art knows how to insert the DNA according to the invention in an expression vector. He is also familiar with the fact that this DNA can be inserted in combination with a DNA coding for another protein or peptide, so that the DNA according to the invention can be expressed in the form of a fusion protein.

Moreover, the person skilled in the art knows conditions of culturing transformed or transfected cells. He also knows methods of isolating and purifying the protein or fusion protein expressed by the DNA according to the invention.

Another subject matter of the present invention is an antibody directed against an above protein or fusion protein. Such an antibody may be made by common methods. It may be polyclonal or monoclonal. For its production it is favorable to immunize animals - in particular rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody - with an above (fusion) protein or with fragments thereof. Further boosters of the animals may be made with the same (fusion) protein or with fragments thereof. The polyclonal antibody may then be obtained from the serum or egg yolk of the animals. For obtaining the monoclonal antibody animal spleen cells are fused with myeloma cells.

Another subject matter of the present invention is a kit. Such a kit comprises one or more of the following components:

(a) a DNA according to the invention,
(b) a spermatogenesis protein according to the invention (X5L protein),

(c) an antibody according to the invention, and
(d) common auxiliary agents, such as carriers, buffers, solvents, controls, etc.

One or more representatives of the individual components may be present each. As to the individual terms reference is made to the above statements. They apply here correspondingly.

The present invention enables the study of spermatogenesis. An X5L protein may be detected by means of an antibody according to the invention. A relationship may be established between an X5L protein and processes occurring in spermatogenesis. Furthermore, an autoantibody directed against this protein may be detected with an X5L protein. Both detections may be made by common methods, in particular a Western blot, an ELISA, an immunoprecipitation or by immunofluorescence. In addition, the organization and expression of the gene coding for an X5L protein may be detected with a nucleic acid according to the invention. This detection may be made as usual, in particular in a Southern blot, by *in situ* hybridization or by PCR.

Besides, the present invention is also suitable to take steps for inhibiting, or increasing the activity of, an X5L protein in persons. An X5L protein may be inhibited by means of an antibody according to the invention. On the other hand, the amount of an X5L protein in persons can be increased using an X5L protein, in particular after linkage to a protein which is not considered foreign by the body, e.g. transferrin or BSA. The same can also be achieved correspondingly with a nucleic acid according to the invention, in particular a DNA which is controlled by a constitutive promoter or a promoter inducible in certain tissues and after its expression results in the provision of an X5L proteins in the person or in certain tissues, e.g. testicles.

Thus, the present invention relates to means for studying spermatogenesis and influencing it by regulating it. The latter comprises both its activation and its inhibition. In

particular, the present invention provides means serving for diagnosing and treating disorders of spermatogenesis.

Brief Description of the Drawings.

Figure 1 shows the DNA and amino acid sequences of a spermatogenesis protein according to the invention which comprises 325 amino acids (X5L protein). The DNA sequence is a human cDNA.

Figure 2 shows the sequences of a genomic DNA coding for an X5L protein. The DNA originates from the human genome. The cDNA of figure 1 starts at the 739 base pair. An intron is present between base pairs 828 and 1129. A polyadenylation site is found at the 2658 base pair.

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Figure 3 shows the DNA and amino acid sequences of an X5L protein comprising 334 amino acids. The DNA sequence is a mouse cDNA.

Figure 4 shows the sequence of a genomic DNA coding for an X5L protein. The DNA originates from the mouse genome. The cDNA of figure 3 starts at the 445 base pair of figure 4(A). An intron is present between the base pairs 492-1232 of figure 4(A). An intron is present between base pairs 1-1136 of figure 4(B). A polyadenylation sequence is found at the 2306 base pair of figure 4(B).

Figure 5 shows the detection of mRNA of an X5L protein in tissues.

Figure 6 shows the detection of mRNA of an X5L protein in testicles. The presence of such an mRNA is limited to tubuli (figure 6(A)). At a cellular level, mRNA of an X5L protein is present in the stages of primary and secondary spermatocytes (stars) and the round spermatocytes (RS). Mature sperms are characterized by (MS) and spermatogoniums are marked by (arrowheads). Sertoli cells (arrows) and Leydig cells (L) have no mRNA of an X5L protein.

The present invention is explained by the below examples.

Example 1: Detection of mRNA of an X5L protein in tissues

(A)

RNA blots of human tissues such as pancreas, suprarenal medulla, thyroid gland, adrenal cortex, testicles, thymus, small intestine, stomach, fetal brain, fetal lungs, fetal liver, and fetal kidney, obtained from Clontech, are subjected to hybridization. A [α^{32}P]dCTP-labeled X5L protein-specific DNA which lies between base pairs 1073 and 1409 of the DNA of figure 1 is used as hybridization sample. The hybridization is carried out overnight followed by wash steps under common conditions. The blots are also hybridized with a radioactive ß-actin sample for the purpose of control (cf. figure 5).

It turns out that mRNA of an X5L protein is expressed in the most varying tissues. The size of the expressed mRNA is 1.7 or 4.3 kb, which is due to differing polyadenylation signals of the DNA of figure 1. It also shows that the expression of mRNA of an X5L protein is the strongest in testicles.

(B)

An RNA *in situ* hybridization is carried out with mouse-testicle tissue. For this purpose, reference is made to the method by Wilkinson, D.G. (1992), Oxford University Press, New York. Antisense or sense RNA samples are used which correspond to base pairs 5-1169 of the DNA of figure 1.

It shows that a strong expression of mRNA of an X5L protein takes place in testicle tissue. It also turns out that the expression is limited to tubuli. At a cellular level, an expression of mRNA of an X5L protein shows in the stages of the primary and secondary spermatocytes as well as the round spermatocytes. Spermatogoniums, mature Sertoli cells and Leydig cells, however, show no expression of mRNA of an X5L protein.

Example 2: Production and purification of a spermatogenesis protein according to the invention (X5L protein)

The DNA of figure 1 is provided with BAMHI linkers subsequently cleaved using BamHI and inserted in the BamHI-cleaved expression vector pQE-8 (Quiagen company). The expression plasmid pQE-8/X5L is obtained. Such a plasmid codes for a fusion protein of 6 histidine residues (N terminus partner) and the X5L protein of figure 1 according to the invention (C terminus partner). pQE-8/X5L is used for transforming *E. coli* SG 13009 (cf. Gottesman, S. *et al.*, J. Bacteriol. 148, (1981), 265-273). The bacteria are cultured in an LB broth with 100 µg/ml ampicillin and 25 µg/ml kanamycin and induced for 4 h using 60 µM isopropyl-ß-D-thiogalactopyranoside (IPTG). Lysis of the bacteria is achieved by adding 6 M guanidine hydrochloride, and a chromatography (Ni-NTA resin) is subsequently carried out with the lysate in the presence of 8 M urea in accordance with the instructions from the manufacturer (Qiagen) of the chromatography material. The bound fusion protein is eluted in a buffer with pH 3.5. After its neutralization, the fusion protein is subjected to 18 % SDS polyacrylamide gel electrophoresis and stained using coomassie blue (cf. Thomas, J.O. and Kornberg, R.D., J. Mol. Biol. 149 (1975), 709-733).

It shows that a (fusion) protein according to the invention can be prepared in highly pure form.

Example 3: Production and detection of an antibody according to the invention

A fusion protein of Example 2 according to the invention is subjected to 18 % SDS polyacrylamide gel electrophoresis. After staining of the gel using 4 M sodium acetate an about 37 kD band is excised from the gel and incubated in phosphate-buffered common salt solution. Gel pieces are sedimented before the protein concentration of the

supernatant is determined by SDS polyacrylamide gel electrophoresis which is followed by coomassie blue staining. Animals are immunized as follows using the gel-purified fusion protein.

Immunization protocol for polyclonal antibodies in rabbits

35 µg of gel-purified fusion protein in 0.7 ml PBS and 0.7 ml of complete or incomplete Freund's adjuvant are used per immunization:

Day 0: 1st immunization (complete Freund's adjuvant)
Day 14: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 28: 3rd immunization (icFA)
Day 56: 4th immunization (icFA)
Day 80: bleeding to death.

The rabbit serum is tested in an immunoblot. For this purpose, a fusion protein of Example 1 according to the invention is subjected to SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10 (1984), 203-209). The Western blot analysis was carried out as described in Bock, C.-T. *et al.*, Virus Genes 8, (1994), 215-229. For this purpose, the nitrocellulose filter is incubated with a first antibody at 37°C for one hour. This antibody is the rabbit serum (1:10000 in PBS). After several wash steps using PBS, the nitrocellulose filter is incubated with a second antibody. This antibody is an alkaline phosphatase-coupled monoclonal goat anti-rabbit IgG antibody (Dianova company) (1:5000) in PBS. 30 minutes of incubation at 37°C are followed by several wash steps using PBS and subsequently by the alkaline phosphatase detection reaction with developer solution (36 µM 5'-bromo-4-chloro-3-indolylphosphate, 400 µM nitro blue tetrazolium, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature until bands become visible.

It shows that polyclonal antibodies according to the

invention can be prepared.

Immunization protocol for polyclonal antibodies in chickens

40 µg of gel-purified fusion protein in 0.8 ml PBS and 0.8 ml of complete or incomplete Freund's adjuvant are used per immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
Day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 50: 3rd immunization (icFA)

Antibodies are extracted from egg yolk and tested in a Western blot. Polyclonal antibodies according to the invention are detected.

Immunization protocol for monoclonal antibodies in mice

12 µg of gel-purified fusion protein in 0.25 ml PBS and 0.25 ml of complete or incomplete Freund's adjuvant are used per immunization. The fusion protein is dissolved in 0.5 ml (without adjuvant) in the 4th immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
Day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 56: 3rd immunization (icFA)
Day 84: 4th immunization (PBS)
Day 87: fusion.

Supernatants of hybridomas are tested in a Western blot. Monoclonal antibodies according to the invention are identified.